experiment with aquarium-reared snails has since shown, however, that penetration of miracidia into *A. glabratus* can occur in total darkness, conjectures to the contrary(5) notwithstanding.

It is of interest that schistosome-infected snails grew somewhat more rapidly than uninfected ones under sterile conditions. Parasitization by certain larval trematodes is said to cause excessive growth of some snails (6, for review), and it has been suggested that such snails apparently overcompensate by voracious feeding for nutrients supposedly withdrawn by the parasite(7). However, under the bacteriologically sterile conditions of the present study, parasitized and control snails were fed the same amount of food per week and little or no food remained unconsumed in either case. If the parasites were "withdrawing" food, parasitized snails might have been expected to be smaller than control snails, while in fact the converse was observed. It seems doubtful that "parasitic castration" could have been significant in this since most of the snails were small and sexually immature, and since the differences in growth become apparent within a week of infection.

Successful cultivation *in vitro* of schistosome-infected *A. glabratus* opens new areas for investigation, particularly insofar as the bacteriologically sterile nature of the system provides a source of uncontaminated larval stages, including cercariae, for possible use as antigens or for studies on cultivation *in vitro*.

Summary. *Australorbis glabratus,* reared axenically to 2-7 mm before exposure, were found to be highly susceptible to infection with *Schistosoma mansoni* in the absence of demonstrable bacteriological contamination. Larval development was normal in all observable respects, and cercariae infectious for mice were produced after normal periods of incubation. Maintenance of these snails in the dark for extended periods did not inhibit larval development. Mortality was very low among schistosome-infected snails maintained in the sterile state. Infected snails tended to grow more rapidly than uninfected snails when both were fed identical rations under controlled, bacteriologically sterile conditions.

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Effects of Amethopterin on Cell Growth and Viral Synthesis.* (26089)

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Mammalian cells have been found not to require any purine or pyrimidine for growth in Eagle's medium(1,2), but the presence of some form of folic acid is obligatory(3). A variety of cultivated mammalian cells have been found unable to proliferate in growth media containing amethopterin (4-amino-N^{10}-methylpteroyl-glutamic acid) an anti-

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folic antagonist(1). The growth inhibitory effects of amethopterin, however, can be completely reversed with a mixture of adenine, glycine and thymidine(4,5). In the present investigation cell growth and viral synthesis was examined in cultures of HeLa cells made thymine-deficient by treatment with amethopterin supplemented only with adenine and glycine.

Materials and methods. The line of HeLa cells was originally obtained from Difco Laboratories, and routinely grown in Eagle's basal medium (EBM)(6) containing 10% human serum. Monolayer cultures for experiments were established in EBM and 10% equine serum (EBM10ES). On day 2 (2 days after initiation of cultures) cultures were washed with Hank's balance salt solutions, and the experimental medium introduced. This medium was renewed on day 4. On day 6 measurements of cell growth were made or the cultures were infected with one of the various viruses studied. The basic experimental medium consisted of EBM10ES supplemented with adenine (1 x 10^{-6}M) and glycine (3 x 10^{-4}M). To this medium, amethopterin (1 x 10^{-6}M) and/or thymidine (3 x 10^{-3}M) was added. Viruses included in this study were poliovirus type 1 (Mahoney), herpes simplex virus (Armstrong), and vaccinia virus (Mich. Dept. of Health). Poliovirus was titrated in tube cultures of HeLa cells and the 50% endpoint calculated by the method of Reed and Muench(7). Both vaccinia and herpes simplex viruses were assayed on chorioallantoic membranes of embryonated eggs according to the method of Beveridge and Burnet(8), and amount of virus present expressed as pock-forming units (PFU) per ml. Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) fractions of the cell cultures were obtained according to the method of Schmidt and Thannhauser (9), and their phosphorus content determined according to the method of Fiske and Subbarow(10). Cellular protein was measured by the method described by Oyama and Eagle(11).

Results. Growth of Thymine-deficient cells. The effect of varying concentrations of amethopterin (AM) in presence of added adenine and glycine on growth of HeLa cells is shown in Table I. Cell growth was determined on the fourth day after introduction of the experimental media, and is expressed in terms of protein content of the cultures.

TABLE I. Growth of HeLa Cells as a Function of Amethopterin (AM) Concentration.

Concentrations of AM (M)	Experimental media	Day	Protein (μg)
0	—	2	340.1
0	—	6	635.6
10^{-8}	A, G	6	625.7
10^{-8}	A, G, T	6	626.6
10^{-6}	A, G	6	373.7
10^{-6}	A, G, T	6	611.7
10^{-4}	A, G	6	387.3
10^{-4}	A, G, T	6	609.9

Cell cultures cultivated in EBM10ES at 37°C for 2 days, then changed to experimental media and reincubated for another 4 days, when protein determinations were made. Experimental media: EBM-10ES with A = adenine (1×10^{-5}M), G = glycine (3×10^{-4}M), and/or T = thymidine (3×10^{-3}M).

AM concentrations of 10^{-6} and 10^{-4} but not at 10^{-8}M, inhibited cell growth (Table I). Degree of inhibition at the 2 higher concentrations of AM was essentially the same. As shown in the same table, this inhibition was removed by addition of thymidine. In other experiments, addition of thymidylic acid instead of thymidine reversed the effect of AM. These results are in agreement with those found by Hakala and Taylor(5) for amethopterin, and by DeMars and Hooper(12) for aminopterin-treated HeLa cells.

In Table II are shown results of an experiment where determinations of cell number, RNA-P and DNA-P, as well as protein, were made.

The AM inhibition of cell growth is again apparent on day 6 if number of cells present in thymine-deficient cultures is compared with that of untreated or thymidine supplemented cultures. There is, in fact, a loss of cells in the thymine-deficient cultures at 6 days as compared to the untreated ones at day 2. This inhibition of cellular multiplication and loss of cells also has been observed by DeMars and Hooper(12) in aminopterin-treated cultures.

TABLE II. "Unbalanced" Growth of HeLa Cells in Presence of Amethopterin.

Experimental media	Day	Cell count $\times 10^6$	mg DNA-P $\times 10^{-10}$/cell	mg RNA-P $\times 10^{-10}$/cell	mg protein $\times 10^{-8}$/cell
—	2	3.32	16.0	48.1	44.1
—	6	6.23	16.8	44.2	41.5
AM, A, G	6	2.23	17.5	95.6	73.0
AM, A, G, T	6	5.95	17.1	45.3	43.4

HeLa cells were cultivated in EBM10ES.

After 2 days, the experimental media were added—EBM10ES with A = adenine (1×10^{-5} M), G = glycine (3×10^{-4}M), and/or T = thymidine (3×10^{-5}M), AM = amethopterin (1×10^{-6}M).

Cell count, protein and nucleic acid determinations were made on 4th day after introduction of experimental media.

The amount of DNA per cell is the same in all cultures, including those deficient in thymine (Table II). However, the deficient cultures have relatively more RNA and protein per cell.

These biochemical observations are supported by direct microscopic examination of the thymine-deficient cultures (Fig. 1). As compared to untreated or thymidine supplemented cultures, those starved of thymine show fewer cells and an absence of mitotic figures. These cells and their nuclei are markedly enlarged. The nuclear chromatin forms a coarse reticulum as compared to the more diffuse arrangement seen in the untreated cells. The nucleoli are enlarged and intensely basophilic.

Ability of thymine-deficient cells to support viral synthesis. Replicate cultures of AM-treated, AM-thymidine-treated, and non-treated HeLa cells were prepared according to the manner described above. These cultures were infected with a sufficiently large inoculum of vaccinia virus, herpes sim-

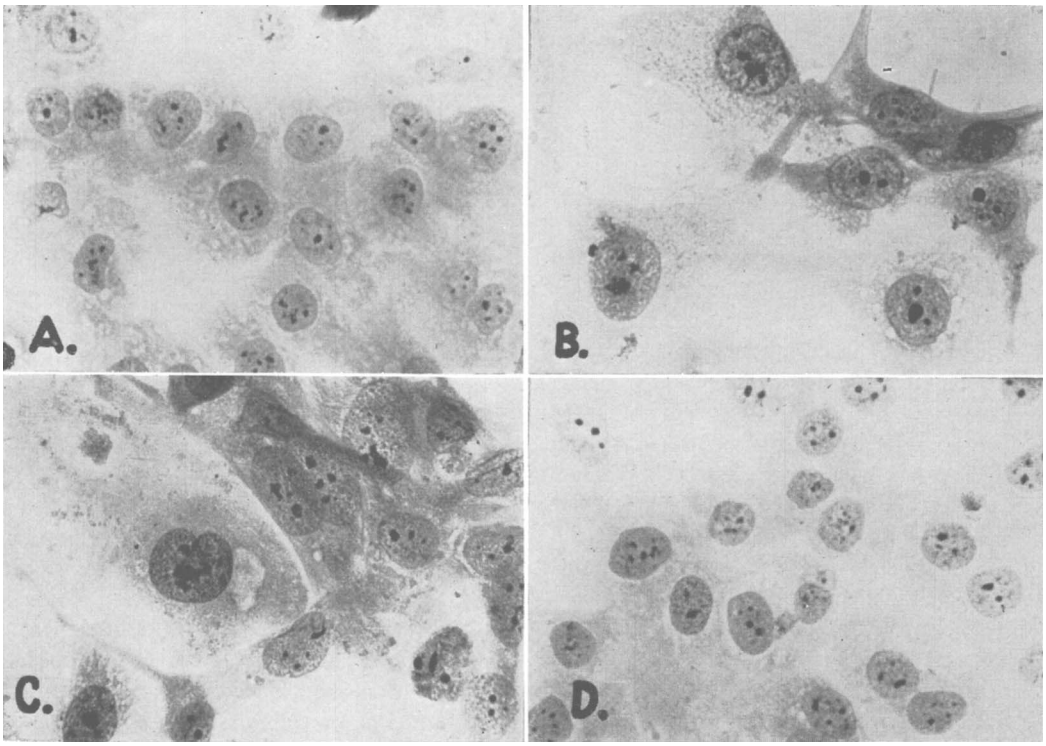

FIG. 1. A. Six day untreated HeLa cell culture. Giemsa. ×440. B. and C. Six day HeLa cell culture, treated from day 2 with AM. Giemsa. ×440. D. Six day HeLa cell culture, treated from day 2 with AM and thymidine. Giemsa. ×440.

TABLE III. Viral Synthesis in Thymine Deficient HeLa Cells.

Virus*	Control	Amethopterin treated	Amethopterin + thymidine treated
Vaccinia†	2.4×10^7§	3×10^6	1.8×10^7
Herpes simplex†	1.92×10^7§	1.7×10^6	2.61×10^7
Poliovirus Type 1‡	$10^{6.5}$‖	$10^{6.2}$	$10^{6.3}$

* Cell cultures incubated with vaccinia and herpes simplex viruses for 4 hr, and poliovirus for 1 hr at 37°C, then washed with Hank's balance salt solution to remove unadsorbed virus and reincubated after addition of experimental media. Total amount of virus was determined at 24 hr.

† Inoculated with approximately 60 PFU/cell.

‡ Inoculated with approximately 10 $TCID_{50}$/cell.

§ Pock-forming units/ml (PFU).

‖ Tissue culture infectious dose ($TCID_{50}$).

plex virus (Armstrong) or poliovirus type 1 (Mahoney) to insure infection of essentially all the cells. Total virus was determined 24 hours after initiation of infection.

The results shown in Table III indicate that in HeLa cells made thymine-deficient by AM, there was inhibition of viral synthesis of both vaccinia and herpes viruses, but not of poliovirus. As compared to the untreated infected control, inhibition of vaccinia and herpes simplex viruses amounted to 87.5% and 91.1% respectively. This inhibition was effectively removed by addition of thymidine to the AM-treated cultures. Although the synthesis of vaccinia virus was inhibited, these cultures showed cytopathic charges resembling those of virus-infected and non-treated cultures. Since the untreated herpes infected cultures showed little or no cytopathology at 24 hours, no definite effect on cytopathic response could be ascribed to the thymine-deficient state. Poliovirus induced cytopathology was the same in all 3 experimental cultures.

Discussion. In the present paper the effect of amethopterin, a folic acid antagonist, on growth of HeLa cells has been examined. Using conditions similar to those of Hakala and Taylor(5), it was found that the activity of this inhibitor can be restricted to a single metabolic pathway. That is, in a medium containing amethopterin supplemented with adenine and glycine, thymine synthesis and cellular multiplication are prevented. This inhibition can be reversed by thymidine or thymidylic acid. As observed here and by Hakala and Taylor(5), the thymine-deficient cultures synthesize less protein than normal. However, when the amount of protein, RNA and DNA are expressed on a per cell basis, it is seen that whereas protein and RNA continue to accumulate, DNA does not. This disproportionate increase in both protein and RNA relative to DNA is reminescent of the phenomenon of "unbalance growth" found in thymineless mutants of bacteria(13). DeMars and Hooper(12), relating total cellular protein to cell number, have recently reported similar results in HeLa cells made thymine-deficient with another folic acid antagonist aminopterin (4-aminopteroylglutamic acid).

The replication of several animal viruses in amethopterin-induced thymine deficient cells was studied and found to be dependent upon the type of nucleic acid contained in the virus. With DNA-containing viruses such as vaccinia and herpes simplex, viral synthesis is inhibited. The inhibition of viral synthesis is prevented by the presence of added thymidine or thymidylic acid. This suggests that the pathways of synthesis of viral or cellular DNA are quite similar, and once again emphasizes the qualitative similarities in the metabolism of virus-infected and uninfected mammalians cell(14). It further suggests, as postulated by Magee, Sheek, and Burrows, (15) that viral DNA is synthesized *de novo*. The inhibition of herpes simplex virus synthesis is of particular interest since the results further support the belief that it is a DNA virus. Newton and Stoker(16), have found in herpes-infected cells increased amounts of DNA which is believed to be non-viral. This increase in DNA content was observed not to occur in the inhibitor-treated herpes-infected cultures (to be reported).

Although the inhibitor prevented vaccinia virus synthesis, it did not interfere with the development of cytopathology. This may be attributable to the "toxic effect" described by Brown, Mayyasi, and Officer(17) for a strain of vaccinia virus. It is also possible that the

action of this antagonist is similar to that of fluorophenylalanine in poliovirus-infected cells, where the amino acid analogue inhibited the synthesis of poliovirus, but not the occurrence of cytopathology(18).

With the RNA-containing poliovirus, no inhibition of virus synthesis or development of cytopathology was observed at concentrations of amethopterin that inhibited DNA synthesis. Thus it appears that active cellular DNA synthesis is not required in the synthesis of a RNA-containing virus.

Summary. Amethopterin, a folic acid antagonist, has been found to prevent multiplication of HeLa cells in Eagle's medium containing added adenine and glycine. This inhibition can be removed by addition of thymidine or thymidylic acid. The amethopterin-induced thymine deficiency specifically results in a cessation of cell division and a disparate increase in cellular RNA and protein to DNA content. Ability of amethopterin-induced thymine deficient cultures to support viral synthesis appears to be dependent upon the type of nucleic acid contained in the virus. With DNA-containing viruses such as vaccinia and herpes simplex, viral replication is inhibited, whereas the synthesis of a RNA-containing virus such as poliovirus is not.

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Electrophoretic Studies of Serum Proteins and Glycoproteins During Early Stages of Experimental Tuberculosis.* (26090)

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Several studies have employed filter paper electrophoresis to determine changes in concentration of serum glycoproteins and proteins in clinical(1,2) and experimental(3) tuberculosis. Previous investigations have been primarily concerned with alterations in serum glycoprotein and protein patterns in well-established infections, and with the effects of therapy. The present study was undertaken to investigate the rate and extent of change in electrophoretic patterns in the early stages of an experimental infection.

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