order of 30 mv. If further inhibition of the Cl^- mechanism occurs, the P.D. will be even less, and with a maintained H^+ rate, and virtual absence of Na^+ and Cl^- transport, the P.D. will be inverted. Thus in the cat, both Na^+ and Cl^- systems are equally suppressed, whereas in guinea pig, the Na^+ mechanism is abolished *in vitro*, according to the above hypothesis.

In SO_4'' conditions, guinea pig mucosa behaves as if the H^+ mechanism is predominant, and as if there is very little net Na^+ or SO_4'' transport, since the P.D. is inverted with only a low H^+ rate.

The effect of Na^+ removal was also of interest. Removal of Na^+ from nutrient side alone resulted in fall of P.D. to zero, with maintained H^+ rate. Under these conditions guinea pig gastric mucosa behaves somewhat similarly to frog mucosa with Na^+ removed from both sides(5). As previously discussed, this is interpreted most simply as a unitary H^+ mechanism under these conditions, whereas in SO_4'', the H^+ mechanism acts as if it is electrogenic. With Na^+ absent from both sides of the mucosa the guinea pig showed an inverted P.D. that was dependent on a maintained H^+ rate. Na^+ readmission reversed those effects. It seems therefore, that in the absence of Na^+ guinea pig gastric mucosa acts as if the H^+ mechanism is predominant and electrogenic, *i.e.*, similar to SO_4'' conditions. Stated alternatively, Na^+ appears essential for Cl^- transport in this system.

Summary. The secreting *in vitro* guinea pig gastric mucosa showed an H^+ rate of 3 μE cm^{-2} hr^{-1}, and an I_{sc} of 57 μa cm^{-2} hr^{-1}. The latter was accounted for by net N→S Cl^- transport and H^+ was sensitive to SCN^- and N_2. Substitution of SO_4'' for Cl^-, and choline for Na^+ inverted the P.D. if H^+ was maintained. An explanation for *in vitro* species differences is proposed.

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Susceptibility of HeLa Cells in S-Phase to Inhibition of DNA Synthesis by Poliovirus Infection.* (31615)

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Since the original observation that poliovirus infection inhibited DNA synthesis in the host cell(1-3), several investigations have concerned themselves with the competence and availability in the infected cells of the reaction components which yield DNA. These studies were prompted by a view that the virion or some product of its vegetative phase acted directly in the DNA primed polymerase reaction to prevent DNA synthesis(4-6). Observations of this type, of a negative nature to date, fail to explain the mechanism of the viral inhibitory effect.

It has also been suggested that the viral action might be directed to some cellular control mechanism that normally governs the rate and sequence of macromolecular synthesis in the uninfected cell(7-9). DNA synthesis in HeLa cells is restricted to approximately 5 hours of the 18-hour reproductive cycle of the cell(10). Conditions which would act to prevent cells from entering the S-phase (DNA productive phase) would inhibit DNA synthesis without acting directly upon the

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DNA polymerase reaction. Thus the virus effect may be directed only against those cells which are not in the process of synthesizing DNA. In cultures where cells are not replicating synchronously, DNA synthesis would not be inhibited completely by this site of attack until the fraction of the population which is in the productive phase at the time inhibition is imposed has duly completed its activity. Such a delayed effect would be consistent with the observation that inhibition of DNA synthesis in HeLa cells is frequently not complete until 4 to 5 hours after infection with poliovirus. Further, with a DNA virus, as vaccinia, where host-cell synthesis of DNA is also blocked, such a mechanism would account for the specific inhibition of host synthesis while viral DNA synthesis proceeds.

The present experiments are designed to test the above hypothesis by determining whether or not infection with poliovirus inhibits promptly DNA synthesis in cells which have been arrested in the S-phase for the first 4 hours of infection.

Materials and methods. Cells. HeLa cells were grown at 37°C in a medium composed of 10% calf serum and twice concentrated Eagle's basal medium(11). They were passaged every 7 days and, periodically, cells and fluid were inoculated into special media to ensure that they were free of mycoplasma and bacteria(12). For experiments which involved isolation of DNA and autoradiography, cells were implanted into Roux bottles containing two cover slips which rested on the wall of the vessel. During the experimental period the calf serum of the medium was reduced to one per cent.

Virus. The Mahoney strain of type 1 poliovirus was passaged routinely in HeLa cells, washed and concentrated by centrifugation and used as a suspension in phosphate-buffered (pH 7.2) saline (PBS). The virus was assayed, by means of plaque assay, on monolayers of HeLa cells in 2 oz prescription bottles, and concentration was expressed in plaque-forming units (PFU).

DNA. DNA was extracted from HeLa cells by the method of Colter *et al*(13) and isolated from the aqueous phase after precipitation of RNA (20% ethanol, V/V) as described by Martinez-Segovia *et al*(14). DNA so isolated was dissolved in saline, precipitated with cold trichloroacetic acid, and dissolved in another portion of the same acid with heating. It was then used to determine the incorporation of H^3-dCMP (H^3-deoxycytidylate). DNA was quantitatively determined by the method of Burton(15).

Autoradiography. Upon removal of the medium from Roux bottles at the end of the experiment, the monolayer was washed with cold PBS. The cover slips were withdrawn, washed 3 times with cold Hanks' balanced salt solution, and placed in cold fixative, consisting of 80% ethanol, glacial acetic acid and neutralized formalin (19:1:1) for 30 minutes. They were then immersed in 70% ethanol. After fixation the cover slips were mounted cell-side up on microscope slides by means of Harleco Synthetic Resin mounting medium (Hartman-Leddon Co., Philadelphia, Pa.). As previously described(16), the cover slips were then coated with liquefied photographic emulsion, stored for 3 days and developed. For purposes of determining the percentage of the total population of cells incorporating isotope, the entire length and width of each cover slip was scanned under oil immersion. Approximately 1200 cells were counted and observed for nuclear silver grains.

Radioactivity. For measurement of radioactivity, 0.1 to 0.5 ml of sample was added to an appropriate volume of scintillation fluid to give a final volume of 10 ml and counted for 10 minutes in an automatic Ansitron Model 1300 liquid scintillation counter.

Sources. FUDR (fluorouridine deoxyribose) Cancer Chemotherapy National Service Center; H^3-deoxycytidine monophosphate, 3.7 c/mmole (Schwarz BioResearch, Inc., Orangeburg, N. Y.).

Experimental and results. Sixteen hours prior to infection with poliovirus, cultures of HeLa cells were treated with a concentration of FUDR which blocks the synthesis of thymidylic acid and thus prevents synthesis of DNA and incorporation of the other deoxyribonucleotides into DNA. Cells in S-phase at the time of addition are thus arrested there while others continue to develop and accu-

TABLE I. Comparative Effect of Poliovirus on DNA Synthesis of Ordinary HeLa Cells and Those Pretreated with Fluorodeoxyuridine.

Additions†	Incorporation* of H^3-dCMP			
	Without FUDR		With FUDR	
	c/m/μg DNA	% Cells (+)	c/m/μg DNA	% Cells (+)
Without thymidylate:				
Control	35.3	24.7	1.5	6.9
Virus	9.0	—	1.7	5.2
With thymidylate:				
Control	39.7	25.6	78.2	53
Virus	12.0	6.6	22.2	15

* Incorporation of isotope, recorded for the interval 4 to 5 hr after virus addition, is expressed as c/m/μg DNA (counts per minute per microgram DNA) and also as percentage of cells whose nuclei show silver grains in autoradiographs.

† Concentrations and times of additions to the cultures were: FUDR (fluorodeoxyuridine), 10^{-6} M at 0 time; virus, 1.2×10^9 PFU/bottle at 16 hr; thymidylate, 40 μg and H^3-dCMP (H^3-deoxycytidine monophosphate), 3 μcuries at 20 hr. Experiment was terminated at 21 hr.

mulate in the S-phase fraction of the culture prior to infection. The continued presence of FUDR in the first hours of infection allows the development of the viral inhibitory apparatus while preventing the cells in S-phase from completing their synthesis of DNA. Addition of thymidylate along with H^3-dCMP at the fourth hour should bypass the block in thymidylate synthesis, allowing an opportunity for incorporation of H^3-dCMP into DNA and thus testing whether DNA synthesis in S-phase cells is sensitive to viral action.

The growth medium was removed from 8 cultures of HeLa cells which had been implanted in Roux bottles containing cover slips. To each Roux bottle 60 ml of fresh medium (twice concentrated Eagle's medium with one per cent calf serum) was added. One-half of the cultures received 0.1 ml of FUDR (0.6×10^{-3} M) while the second half received 0.1 ml of PBS (phosphate buffered saline, pH 7.2). After 16 hours further incubation at 37°C, 3 ml of PBS containing poliovirus ($.4 \times 10^9$ PFU/ml) was added to two cultures of each group. The incubation was continued an additional 4 hours whereupon 3 μc of H^3-dCMP were introduced into all cultures. Half of those containing FUDR received thymidylate at this time. The experiment was terminated after one additional hour. The cover slips were removed and prepared for autoradiography. The remaining cells of the culture were extracted with phenol, their DNA isolated and the level of incorporation of tritium determined.

In Table I, where the treatment of each culture is indicated, the degree of incorporation of H^3-dCMP into DNA is recorded, as well as the fraction of cells in the culture which incorporated the isotope. The incorporation of H^3-dCMP into DNA of the HeLa cell was 96% inhibited by FUDR. Addition of thymidylate to cultures untreated with FUDR affected the incorporation in no significant way. However, addition of thymidylate to cultures treated with FUDR for 16 hours promptly restored the incorporation of isotope into DNA at 197% of the rate in cultures lacking FUDR, *i.e.*, 78.2 c/min/μg DNA compared to 39.7. While 24.7% of cells in the untreated culture were participating in DNA synthesis (autoradiographic data), the fraction in the FUDR treated culture was 6.9% and, in the first hour following thymidylate addition, rose to 53%.

Cultures untreated with FUDR incorporated H^3-dCMP to level of 12 c/min/μg DNA in the interval 4 to 5 hours after infection compared to 39.7 for the uninfected corresponding control. Infected cultures which contained FUDR and received thymidylate at the fourth hour incorporated the isotope at a level of 22.2 compared to 78.2 for the comparable uninfected culture. This represents an inhibition due to infection of 70% in the former case and 72% in the latter. The autoradiographic data indicate in each case, with or without pretreatment with FUDR, that the effect of infection is to re-

duce the fraction of cells incorporating isotope. The reductions of 75 and 72%, respectively, closely parallel the results of chemical analysis.

Discussion. The data indicate that the HeLa cells can be arrested in the S-phase by the use of FUDR, and that those cells not synthesizing DNA continue in their development and accumulate in the S-phase fraction, thus producing an increased degree of synchronous replication of the cells in culture. In the particular experiment described here, all cells are not in the S-phase. After 16 hours treatment with FUDR, the value may range from 50 to 75%. The viral induced apparatus for inhibition of DNA synthesis seems to develop in the usual manner in infected cells which are not synthesizing DNA, *i.e.,* in the presence of FUDR. Upon addition of thymidylate to such cells, inhibition is a prompt event or results from a previous event which only now becomes manifest in the inability of the H^3-dCMP to be incorporated into DNA. Even though an increased fraction of cells was arrested in the S-phase at the time of infection, the degree of inhibition observed upon addition of thymidylate was the same as in cultures where the S-phase was proceeding during the early stages of infection. Viral inhibition of DNA synthesis results not from preventing cells from entering the DNA productive phase but rather by action upon those cells in culture which are in the process of synthesizing DNA. However, since the development of the viral inhibitory apparatus does not seem to be triggered by the actual process of DNA synthesis, the data suggest that it develops also in those cells that normally are not synthesizing DNA.

Summary. The inhibition of DNA synthesis in the 4 to 5 hour interval following infection with poliovirus was the same in untreated HeLa cells as in those whose DNA synthesis had been blocked up to this point by action of FUDR. The viral mechanism which inhibits DNA synthesis can develop in the absence of DNA synthesis. The inhibitory action is directed against cells already in the DNA productive phase rather than to prevent cells from entering this phase.

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